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C07K 7/10, C12N 15/00, 15/82, A61K 37/02

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08/003,884 13 January 1993 (13.01.93) US

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(74) Agents: ROTH, Michael, J. et al.; 700 Capital Square, 400 Locust Street, Des Moines, IA 50309 (US).

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

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(54) Title: SYNTHETIC AMPHIPATHIC PEPTIDES WITH ANTIMICROBIAL ACTIVITY

(57) Abstract

Synthetic polypeptides exhibiting amphipathic alpha-helices provide cell-expressible antimicrobial activity.

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INTERNATIONAL SEARCH REPORT

Interna[illegible]al Application No
PCT/US 94/00383

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C07K7/10 C12N15/00 C12N15/82 A61K37/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,92 01462 (THE SCRIPPS RESEARCH INSTITUTE) 6 February 1992 * whole disclosure * ---	1-20
A	WO,A,92 18146 (THE CHILDREN'S HOSPITAL OF PHILADELPHIA) 29 October 1992 * whole disclosure * ---	1-20
A	EP,A,0 182 278 (WAKUNAGA SEIYAKU) 28 May 1986 * whole disclosure * ---	1-20
A	EP,A,0 502 718 (PIONEER HI-BRED INT.) 9 September 1992 * whole disclosure * --- -/--	1-20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 October 1994	18. 10. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Hermann, R

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/US 94/00383

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE EMBO JOURNAL vol. 9, no. 1 , 1990 pages 217 - 224 KYLSTEN, P. ET AL. 'The cecropin locus in drosophila' * figure 3 * -----	1-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00383

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see annex

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

1-20 all partially

(subject 1,2,3)

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

1.- Claims 1-20, all partially: Peptides having the C-terminal sequence AGPGVTIGIAHAKSQLW (= SEQ.I.D. Nos. 1-8), and all subject-matter relating to such peptides.

2.- Claims 1-20, all partially: Peptides having the sequence GWLRRIGRRIERVGQH (SEQ.I.D. Nos. 9,11,12), and all subject-matter relating to such peptides.

3.- Claims 1-20, all partially: Peptides having the sequence LKKALRALARHWK (SEQ.I.D. Nos 10-12), and all subject-matter relating to such peptides.

4.- Claims 1-120, all partially: Peptides having the C-terminal sequence ALMGEAVQT (= SEQ.I.D. Nos 13-15), and all subject-matter relating to such peptides.

INTERNATIONAL SEARCH REPORT

...ormation on patent family members

Interna... ...al Application No
PCT/US 94/00383

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9201462	06-02-92	JP-T-	6504260	19-05-94
		US-A-	5294605	15-03-94
WO-A-9218146	29-10-92	US-A-	5225399	06-07-93
		AU-B-	652261	18-08-94
		AU-A-	1914992	17-11-92
		EP-A-	0544860	09-06-93
		JP-T-	6500573	20-01-94
EP-A-0182278	28-05-86	JP-A-	61122299	10-06-86
EP-A-0502718	09-09-92	AU-A-	1139992	10-09-92

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SYNTHETIC AMPHIPATHIC PEPTIDES WITH ANTIMICROBIAL ACTIVITY

TECHNICAL FIELD

This invention relates to synthetic peptides which have antimicrobial activity.

BACKGROUND OF THE INVENTION

Disease resistance is an important objective of the genetic engineering of crop plants. Numerous fungi and bacteria are serious pests of common agricultural crops. The maize plant is susceptible to a variety of pathogenic fungi that reduce yield and quality of the crop all over the world. In the United States alone annual losses in the Corn Belt range from about 7% to about 17%. One method of controlling diseases has been to apply antimicrobial organic or semiorganic chemicals to crops. This method has numerous, art-recognized problems. A more recent method of control of microorganism pests has been the use of biological control organisms which are typically natural competitors or inhibitors of the troublesome microorganisms. However, it is difficult to apply biological control organisms to large areas, and even more difficult to cause those living organisms to remain in the treated area for an extended period. Still more recently, techniques in recombinant DNA have provided the opportunity to insert into plant cells cloned genes which express antimicrobial compounds. This technology has given rise to additional concerns about eventual microbial resistance to well-known, naturally occurring antimicrobials, particularly in the face of heavy selection pressure, which may occur in some areas. Thus, a continuing effort is underway to express naturally occurring antimicrobial compounds in plant cells directly by translation of a single structural gene.

However, there is a limited pool of naturally occurring peptides and other compounds with which molecular biologists

can work. Attention is now focused on the rational design of entirely new peptides which can function effectively in plant cell expression systems and in other uses where antimicrobial peptides can be used.

In addition, there are other aspects of plant cell expression systems which make the design of new antimicrobial peptides desirable. Crop plants have more important things to do than fight disease. They are sources of sugars, starches, proteins, oils, fibers, and other raw materials. Genetic engineers would also like to modify, and often to enhance, the production of those natural plant products. Unfortunately, plant cells can only produce large quantities of a few cellular components at a time. If they are producing high levels of storage proteins, it is difficult for them to also produce high levels of antifungal compounds. Thus, genetic engineers face a quandary in designing advanced plant systems which require high-level expression of multiple genes. The creation of entirely new antimicrobial peptides offers the molecular designer the opportunity to select structures which enhance the plant's content of various important or limiting amino acids while also providing antimicrobial activity. One example of this is the copending application of Rao and Beach, "High Lysine Derivatives of Alpha-Hordothionin", No. ____, filed January 13, 1993. Even so, there continues to exist a need for still more compounds which can be evaluated and used in various plant and non-plant antimicrobial applications.

The principle of amphipathy has been used in the past to design biologically active proteins. In 1981 De Grado et al., J.Am.Chem.Soc. 103:679-681 showed that the completely synthetic analog of melittin was biologically active even though it had no homology to the natural peptide. Fink et al., Int.J.Pep.Prot.Res. 33: 412-421 (1989) and Boman et al., FEBS Lett. 1: 103-106 (1989) have demonstrated antibacterial activity of synthetic cecropin-like model peptides and cecropin-melittin hybrid compounds. Lee et al., Biochem.Biophys.Acta 862: 211-219 (1986) and Agawa et al., J.Biol.Chem. 266: 20218-20222 (1991) have shown a relationship between antimicrobial activity and amphiphilic properties of

basic model peptides. More recently, Moser, Protein Eng. 5: 323-331 (1992) has reported on the design, synthesis and structure of an amphipathic peptide with pH-inducible hemolytic activity. Taylor et al., Molec.Pharm. 22: 657-666 (1982) have synthesized analogs of beta-endorphin possessing complete biological activity. Frohlich and Wells, Int.J.Pep.Prot.Res. 37: 2-6 (1991) have suggested the idea of peptide amphipathy in the design of mechanism-based insecticides.

DISCLOSURE OF THE INVENTION

It has now been determined that new lytic peptides can be designed to provide antifungal or antimicrobial activity. These proteins can be expressed to enhance resistance to fungal diseases in plants. While not intending to be limited by theory, this discovery is based upon a departure from prior art methods involving de novo synthesis of compounds. Instead, the compounds of this invention were designed according to the principle that, so long as the amphipathic helix secondary structure constitutes the predominant portion of the molecule (i.e., that which determines its physico-chemical behavior), the peptide sequence can be constructed with as much or as little sequence homology as desired to existing bioactive compounds, or with no sequence homology at all to existing bioactive compounds, provided that it has a hydrophobic moment as determined by the Eisenberg algorithm (Eisenberg et al., J.Mol.Biol. 179: 125-142, 1984) which is similar to that of naturally occurring bioactive molecules. In general, this hydrophobic moment can be expected to place them in the SURFACE region of the hydrophobic moment plot of naturally occurring antimicrobial proteins as defined by Eisenberg and colleagues.

The compounds of this invention have amino acid sequences as indicated in SEQUENCE I.D. Nos. 1 through 15. Although there is little or no sequence homology in these peptides at the primary structure level, there is considerable similarity at the secondary structure and hydrophobic moment levels,

which structural similarity is responsible for their similar antimicrobial activities. These peptides are all characterized by a common structural theme that is critical to their lytic activity, namely, regions which form amphipathic alpha helices. In such a helix the hydrophobic amino acid residues are oriented on one face of the helix and the hydrophilic amino acids are oriented on the other face. While not intending to be limited by theory, it appears that this is the structural element which is capable of interacting with and permeabilizing the plasma membranes of a broad spectrum of target organisms, including both bacteria and fungi, eventually leading to cell death.

Comparison of some of the sequences illustrates the principle that design of these synthetic compounds offers considerable flexibility in selection of amino acid and other profiles while retaining antimicrobial activity. SEQUENCE I.D. Nos. 3 and 4 are quite similar, yet several arginine residues in SEQUENCE I.D. No. 3 have been replaced by lysine residues in SEQUENCE I.D. No. 4. The same observation can be made by comparison of SEQUENCE I.D. No. 9 and SEQUENCE I.D. No. 10. Since lysine is a limiting amino acid in maize, this design flexibility offers the opportunity to impart disease resistance to maize with a high-lysine peptide if desired.

Synthesis of the compounds described herein was performed according to methods of peptide synthesis which are well known in the art and thus constitute no part of this invention. In vitro, we have synthesized the compounds on an Applied Biosystems Model 431A peptide synthesizer using FastMoc™ chemistry involving HBtu [2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, as published by Rao et al., Int. J. Pep. Prot. Res. 40:508-515 (1992). Peptides were cleaved following standard protocols and purified by reverse phase chromatography using standard methods. The amino acid sequence of each peptide was confirmed by automated Edman degradation on an Applied Biosystems 477A protein sequencer/120A PTH analyzer. More preferably, however, the compounds of this invention are synthesized in vivo by bacterial or plant cells which have been transformed by

insertion of an expression cassette containing a synthetic gene which when transcribed and translated yields the desired compound. Such empty expression cassettes, providing appropriate regulatory sequences for plant or bacterial expression of the desired sequence, are also well-known, and the nucleotide sequence for the synthetic gene, either RNA or DNA, can readily be derived from the amino acid sequence for the protein using standard reference texts. Preferably, such synthetic genes will employ plant-preferred codons to enhance expression of the desired protein.

Industrial Applicability

The following description further exemplifies the compositions of this invention and the methods of making and using them. However, it will be understood that other methods, known by those of ordinary skill in the art to be equivalent, can also be employed.

Plants

The polypeptides employed in this invention can be effectively applied to plants afflicted with susceptible microorganisms by any convenient means, including spray, creams, dust or other formulation common to the antimicrobial arts. The compound can also be incorporated systemically into the tissues of a treated plant so that in the course of infesting the plant the pathogens will be exposed to antimicrobial amounts of the compound of this invention. One method of doing this is to incorporate the compound in a non-phytotoxic vehicle which is adapted for systemic administration to the susceptible plants. This method is commonly employed with fungicidal materials such as captan and is well within the purview of one of ordinary skill in the art of plant fungicide formulation. However, since the genes which code for these compounds can be inserted into an appropriate expression cassette and introduced into cells of a susceptible plant species, an especially preferred embodiment of this method involves inserting into the genome of the plant a DNA sequence coding for a compound of this invention in

proper reading frame, together with transcription initiator and promoter sequences active in the plant. Transcription and translation of the DNA sequence under control of the regulatory sequences causes expression of the protein sequence at levels which provide an antimicrobial amount of of the protein in the tissues of the plant which are normally infected by the pathogens.

The plant is preferably a plant susceptible to infection and damage by one or more of F. graminearum, Fusarium moniliforme, F. oxysporum, A. flavus, P. medicaginis, Alternaria longipes, Colletototrichum graminicola, Verticillium albo-atrum, Phytophthora megasperme f.sp. glycinea, Macrophomina phaseolina, Diasporthe phaseolorum caulivor, Sclerotinia sclerotiorum, and Sclerotinia trifoliorum. These include corn (Zea mays) and sorghum (Sorghum bicolor). However, this is not to be construed as limiting, inasmuch as these two species are among the most difficult commercial crops to reliably transform and regenerate, and these pathogens also infect certain other crops. Thus the methods of this invention are readily applicable via conventional techniques to numerous plant species, if they are found to be susceptible to the plant pathogens listed hereinabove, including, without limitation, species from the genera Allium, Antirrhinum, Arabidopsis, Arachis, Asparagus, Atropa, Avena, Beta, Brassica, Browallia, Capsicum, Cicer, Cicla, Citrullus, Citrus, Cucumis, Cucurbita, Datura Daucus, Digitalis, Fagopyrum, Fragaria, Geranium, Glycine, Gossypium, Helianthus, Hordeum, Hemerocallis, Lactuca, Lens, Lolium, Lotus, Lycopersicon, Majorana, Manihot, Medicago, Nasturtium, Nicotiana, Oryza, Pelargonium, Persea, Petunia, Phaseolus, Pisum, Ranunculus, Raphanus, Ricinus, Saccharum, Secale, Senecio, Setaria, Solanum, Spinacia, Trifolium, Triticum, Bromus, Cichorium, Hyoscyamus, Linum, Nemesia, Panicum, Onobrychis, Pennisetum, Salpiglossis, Sinapis, Trigonella, and Vigna.

Preferred plants that are to be transformed according to the methods of this invention are cereal crops, including

maize, rye, barley, wheat, sorghum, oats, millet, rice, triticale, sunflower, alfalfa, rapeseed and soybean.

Synthetic DNA sequences can then be prepared which code for the appropriate sequence of amino acids, and this synthetic DNA sequence can be inserted into an appropriate plant expression cassette.

Likewise, numerous plant expression cassettes and vectors are well known in the art. By the term "expression cassette" is meant a complete set of control sequences including initiation, promoter and termination sequences which function in a plant cell when they flank a structural gene in the proper reading frame. Expression cassettes frequently and preferably contain an assortment of restriction sites suitable for cleavage and insertion of any desired structural gene. It is important that the cloned gene have a start codon in the correct reading frame for the structural sequence. In addition, the plant expression cassette preferably includes a strong constitutive promoter sequence at one end to cause the gene to be transcribed at a high frequency, and a poly-A recognition sequence at the other end for proper processing and transport of the messenger RNA. An example of such a preferred (empty) expression cassette into which the cDNA of the present invention can be inserted is the pPHI414 plasmid developed by Beach et al. of Pioneer Hi-Bred International, Inc., Johnston, IA, as disclosed in U.S. Patent Application No. 07/785,648, filed October 31, 1991. Highly preferred plant expression cassettes will be designed to include one or more selectable marker genes, such as kanamycin resistance or herbicide tolerance genes.

By the term "vector" herein is meant a DNA sequence which is able to replicate and express a foreign gene in a host cell. Typically, the vector has one or more endonuclease recognition sites which may be cut in a predictable fashion by use of the appropriate enzyme. Such vectors are preferably constructed to include additional structural gene sequences imparting antibiotic or herbicide resistance, which then serve as markers to identify and separate transformed cells. Preferred markers/selection agents include kanamycin,

chlorosulfuron, phosphonothricin, hygromycin and methotrexate. A cell in which the foreign genetic material in a vector is functionally expressed has been "transformed" by the vector and is referred to as a "transformant."

A particularly preferred vector is a plasmid, by which is meant a circular double-stranded DNA molecule which is not a part of the chromosomes of the cell.

As mentioned above, both genomic and cDNA encoding the gene of interest may be used in this invention. The vector of interest may also be constructed partially from a cDNA clone and partially from a genomic clone. When the gene of interest has been isolated, genetic constructs are made which contain the necessary regulatory sequences to provide for efficient expression of the gene in the host cell. According to this invention, the genetic construct will contain (a) a first genetic sequence coding for the protein or trait of interest and (b) one or more regulatory sequences operably linked on either side of the structural gene of interest. Typically, the regulatory sequences will be selected from the group comprising of promoters and terminators. The regulatory sequences may be from autologous or heterologous sources.

Promoters that may be used in the genetic sequence include nos, ocs and CaMV promoters.

An efficient plant promoter that may be used is an overproducing plant promoter. Overproducing plant promoters that may be used in this invention include the promoter of the small sub-unit (ss) of the ribulose-1,5-biphosphate carboxylase from soybean (Berry-Lowe et al., J. Molecular and App. Gen., 1:483-498 (1982)), and the promoter of the cholorophyll a-b binding protein. These two promoters are known to be light-induced, in eukaryotic plant cells (see, for example, Genetic Engineering of Plants, An Agricultural Perspective, A. Cashmore, Pelham, New York, 1983, pp. 29-38, G. Coruzzi et al., J. Biol. Chem., 258:1399 (1983), and P. Dunsmuir, et al., J. Molecular and App. Gen., 2:285 (1983)).

The expression cassette comprising the structural gene for the protein of this invention operably linked to the desired control sequences can be ligated into a suitable cloning

vector. In general, plasmid or viral (bacteriophage) vectors containing replication and control sequences derived from species compatible with the host cell are used. The cloning vector will typically carry a replication origin, as well as specific genes that are capable of providing phenotypic selection markers in transformed host cells. Typically, genes conferring resistance to antibiotics or selected herbicides are used. After the genetic material is introduced into the target cells, successfully transformed cells and/or colonies of cells can be isolated by selection on the basis of these markers.

Typically, an intermediate host cell will be used in the practice of this invention to increase the copy number of the cloning vector. With an increased copy number, the vector containing the gene of interest can be isolated in significant quantities for introduction into the desired plant cells. Host cells that can be used in the practice of this invention include prokaryotes, including bacterial hosts such as E. coli, S. typhimurium, and Serratia marcescens. Eukaryotic hosts such as yeast or filamentous fungi may also be used in this invention. Since these hosts are also microorganisms, it will be essential to ensure that plant promoters which do not cause expression of the protein in bacteria are used in the vector.

The isolated cloning vector will then be introduced into the plant cell using any convenient technique, including electroporation (in protoplasts), retroviruses, bombardment, and microinjection into cells from monocotyledonous or dicotyledonous plants in cell or tissue culture to provide transformed plant cells containing as foreign DNA at least one copy of the DNA sequence of the plant expression cassette. Preferably, the monocotyledonous species will be selected from maize, sorghum, wheat or rice, and the dicotyledonous species will be selected from soybean, alfalfa, rapeseed, sunflower or tomato. Using known techniques, protoplasts can be regenerated and cell or tissue culture can be regenerated to form whole fertile plants which carry and express the gene for a protein according to this invention. Accordingly, a highly

preferred embodiment of the present invention is a transformed maize plant, the cells of which contain as foreign DNA at least one copy of the DNA sequence of an expression cassette of this invention.

Finally, this invention provides methods of imparting resistance to diseases caused by microorganisms selected from F. graminearum, Fusarium moniliforme, F. oxysporum, A. flavus, P. medicaginis, Alternaria longipes, Colletototrichum graminicola, Verticillium albo-atrum, Phytophthora megasperme f.sp. glycinea, Macrophomina phaseolina, Diasporthe phaseolorum caulivor, Sclerotinia sclerotiorum, and Sclerotinia trifoliorum to plants of a susceptible taxon, comprising the steps of:

a) culturing cells or tissues from at least one plant from the taxon,

b) introducing into the cells or tissue culture at least one copy of an expression cassette comprising a structural gene for one or more of the compounds of this invention, operably linked to plant regulatory sequences which cause the expression of the compound or compounds in the cells, and

c) regenerating disease-resistant whole plants from the cell or tissue culture. Once whole plants have been obtained, they can be sexually or clonally reproduced in such manner that at least one copy of the sequence provided by the expression cassette is present in the cells of progeny of the reproduction.

Alternatively, once a single transformed plant has been obtained by the foregoing recombinant DNA method, conventional plant breeding methods can be used to transfer the structural gene for the compound of this invention and associated regulatory sequences via crossing and backcrossing. Such intermediate methods will comprise the further steps of

a) sexually crossing the disease-resistant plant with a plant from the disease-susceptible taxon;

b) recovering reproductive material from the progeny of the cross; and

c) growing disease-resistant plants from the reproductive material. Wher desirable or necessary, the

agronomic characteristics of the susceptible taxon can be substantially preserved by expanding this method to include the further steps of repetitively:

a) backcrossing the disease-resistant progeny with disease-susceptible plants from the susceptible taxon; and

b) selecting for expression of antimicrobial activity (or an associated marker gene) among the progeny of the backcross, until the desired percentage of the characteristics of the susceptible taxon are present in the progeny along with the gene imparting antimicrobial activity.

By the term "taxon" herein is meant a unit of botanical classification of genus or lower. It thus includes genus, species, cultivars, varieties, variants, and other minor taxonomic groups which lack a consistent nomenclature.

It will also be appreciated by those of ordinary skill that the plant vectors provided herein can be incorporated into Agrobacterium tumefaciens, which can then be used to transfer the vector into susceptible plant cells, primarily from dicotyledonous species. Thus, this invention provides a method for imparting antimicrobial activity and disease resistance in Agrobacterium tumefaciens-susceptible dicotyledonous plants in which the expression cassette is introduced into the cells by infecting the cells with Agrobacterium tumefaciens, a plasmid of which has been modified to include a plant expression cassette of this invention.

Human and Veterinary Pharmaceutical Use

This invention also provides methods of treating and preventing infection by susceptible organisms in a human or lower animal host in need of such treatment, which method comprises administration to the human or lower animal host in need of such treatment a therapeutically effective amount of a polypeptide of this invention or a composition containing one or more of the polypeptides. The polypeptides of the present invention may be administered parenterally, by inhalation spray, rectally or topically in dosage unit formulations containing conventional nontoxic pharmaceutically acceptable

carriers, adjuvants and vehicles as desired. The term parenteral as used herein includes subcutaneous, intravenous, intramuscular, intraarticular and intrathecal injection and infusion techniques. As with other polypeptides, the polypeptides of this invention are not known to be active orally.

Total daily dose of the compounds of this invention administered to a host in single or divided doses may be in amounts, for example, of from 1 to 2000 mg/kg body weight daily and more usually 50 to 500 mg/kg. Dosage unit compositions may contain such amounts or fractions or submultiples thereof as appropriate to make up the daily dose. It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, rate of excretion, drug combination and the severity of the particular disease undergoing therapy.

This invention also provides pharmaceutical compositions in unit dosage form, comprising an effective amount of a compound of this invention in combination with a conventional pharmaceutical carrier. As used herein, the term "pharmaceutical carrier" means a solid or liquid filler, diluent or encapsulating material. Some examples of the materials which can serve as pharmaceutical carriers are sugars, such as lactose, glucose and sucrose; starches such as corn starch and potato starch; cellulose and its derivatives such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients such as cocoa butter and suppository waxes; oils such peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; polyols such as propylene glycol, glycerin, sorbitol, mannitol and polyethylene glycol; esters such as ethyl oleate and ethyl laurate; agar; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution, ethyl alcohol and phosphate buffer solutions, as

well as other non-toxic compatible substances used in pharmaceutical formulations. Wetting agents, emulsifiers and lubricants such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, and perfuming agents and preservatives can also be present in the compositions, according to the desires of the formulator. The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration.

By "therapeutically effective amount" herein is meant an amount of either polypeptide or combination thereof sufficient to provide antimicrobial activity so as to alleviate or prevent infection by susceptible organisms in the human or lower animal being treated at a reasonable benefit/risk ratio attendant with any medical treatment.

Antimicrobial Testing

The antifungal activity of compounds synthesized in accord with this invention was measured using art-recognized methods, as described in Duvick et al., J.Biol.Chem. 26:18814-18820 (1992). Results are as follows:

Table 1

Antifungal Activity of Synthetic Peptides

	A. flavus		F. graminearum		F. moniliforme	
Peptide*	MIC	MCIC	MIC	MCIC	MIC	MCIC
1	NA	NA	17	>40	17	>40
2	NA	NA	14	19	14	39
3	NA	NA	13	26	13	26
4	NA	NA	13	51	17	136
6	80	>80	10	20	15	40
7	40	>80	10	40	10	40
9	15	80	10	20	10	15
10	80	>80	10	20	7.5	10
Cecropin A	80	>80	60	>80	80	>80
Mastoparan	20	40	5	10	2.5	10

Peptides of this invention are identified by their SEQUENCE I.D. Nos.

MIC is the minimum inhibitory concentration, the concentration in μg/ml achieving a score of 1 or greater. MCIC is the Minimum Complete Inhibitory Concentration, the concentration in μg/ml achieving a score of 3 or greater. The ">" symbol indicates that the MIC or MCIC was higher than the highest tested concentration.

Table 2

Antimicrobial Activity Expressed as % Inhibition of Growth

E. coli

	Concentration (μg/ml)					
Peptide	100	50	25	12.5	6.25	3.00
1	15	4	0	0	0	0
2	44	38	35	30	26	8
3		0	0	0	0	0
4		0	0	0	0	0
5		0	0	0	0	0
10		0	0	0	0	0

P. syringae

	Concentration (μg/ml)					
Peptide	100	50	25	12.5	6.25	3.00
1	72	27	10	5	2	0
2	95	92	91	90	90	83
3		52	48	30	11	0
4		32	27	14	3	0
5	100	100	56	30	20	12
10		48	27	19	0	0

E. stewartii

	Concentration (μg/ml)					
Peptide	100	50	25	12.5	6.25	3.00
1		-	-	-	-	-
2		-	-	-	-	-
3		100	80	49	0	0
4		100	100	57	55	54
5		100	100	62	47	42
10		0	0	0	0	0

B. pumilus

	Concentration (μg/ml)					
Peptide	100	50	25	12.5	6.25	3.00
1	77	25	19	9	7	0
2	87	69	22	0	0	0
3	-	-	-	-	-	-
4	-	-	-	-	-	-
5	-	-	-	-	-	-
10	-	-	-	-	-	-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Rao, A. Gururaj; Zhong, Lingxiu

(ii) TITLE OF INVENTION: SYNTHETIC ANTIMICROBIAL PEPTIDES

(iii) NUMBER OF SEQUENCES: 15

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Pioneer Hi-Bred International, Inc.

(B) STREET: 700 Capital Square, 400 Locust Street

(C) CITY: Des Moines

(D) STATE: Iowa

(E) COUNTRY: United States

(F) ZIP: 50309

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette, 3.5 inch, 1.44 Mb storage

(B) COMPUTER: IBM Compatible

(C) OPERATING SYSTEM: MS-DOS, Microsoft Windows

(D) SOFTWARE: Microsoft Windows Notepad

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Roth, Michael J.

(B) REGISTRATION NUMBER: 29,342

(C) REFERENCE/DOCKET NUMBER: 0233 US

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (515) 245-3594

(B) TELEFAX: (515) 245-3634

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(iii) HYPOTHETICAL: No
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:
Lys Lys Ile Glu Lys Ala Ile Lys His Ile Pro Lys Lys Ile Lys Ala Gly Pro Gly Val Thr Ile Gly Ile Ala His Ala Lys Ser Gln Leu Trp

(2) INFORMATION FOR SEQ ID NO: 2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:
Lys Leu Lys Lys Ala Leu Arg Ala Leu Ala Arg His Trp Lys Ala Gly Pro Gly Val Thr Ile Gly Ile Ala His Ala Lys Ser Gln Leu Trp

(2) INFORMATION FOR SEQ ID NO: 3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:
Gln Arg Ala Val Arg Arg Ile Tyr Arg Ala Ile Arg His Ile Pro Arg Arg Ile Arg Ile Arg Ala Leu Ala Gly Pro Gly Val Thr Ile Gly Ile Ala His Ala Lys Ser Gln Leu Trp

(2) INFORMATION FOR SEQ ID NO: 4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Gln Arg Ala Val Lys Lys Ile Glu Lys Ala Ile Lys His Ile Pro
Lys Lys Ile Lys Ile Arg Ala Leu Ala Gly Pro Gly Val Thr Ile
Gly Ile Ala His Ala Lys Ser Gln Leu Trp

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 40 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Ile Gln Arg Val Ala Gln Lys Leu Lys Lys Ala Leu Arg Ala Leu
Ala Arg His Trp Lys Arg Ala Leu Ala Gly Pro Gly Val Thr Ile
Gly Ile Ala His Ala Lys Ser Gln Leu Trp

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 43 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Ile Arg Ala Leu Gln Arg Ala Val Arg His Pro Arg Ala Ile Arg
Arg Ile Tyr Arg Gly Trp Lys Lys Ala Ile Arg Ala Gly Pro Gly
Val Thr Ile Gly Ile Ala His Ala Lys Ser Gln Leu Trp

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 38 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(A) DESCRIPTION: hordothionin derivative

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Lys Leu Ile Arg Lys Leu Ile Arg Trp Leu Arg Arg Lys Ile Arg Ala Leu Gln Arg Ala Val Ala Gly Pro Gly Val Thr Ile Gly Ile Ala His Ala Lys Ser Gln Leu Trp

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 41 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Gln Arg Ala Val Gly Trp Leu Arg Arg Ile Gly Arg Arg Ile Glu Arg Val Gly Gln His Leu Arg Ala Leu Ala Gly Pro Gly Val Thr Ile Gly Ile Ala His Ala Lys Ser Gln Leu Trp

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Arg Arg Ile Tyr Arg Ala Ile Arg His Ile Pro Arg Arg Ile Arg Gly Trp Leu Arg Arg Ile Gly Arg Arg Ile Glu Arg Val Gly Gln His

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Lys Lys Ile Glu Lys Ala Ile Lys His Ile Pro Lys Lys Ile Lys
Leu Lys Lys Ala Leu Arg Ala Leu Ala Arg His Trp Lys

(2) INFORMATION FOR SEQ ID NO: 11:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:
Gly Trp Leu Arg Arg Ile Gly Arg Arg Ile Glu Arg Val Gly Gln
His Lys Leu Lys Lys Ala Leu Arg Ala Leu Ala Arg His Trp Lys

(2) INFORMATION FOR SEQ ID NO: 12:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:
Lys Leu Lys Lys Ala Leu Arg Ala Leu Ala Arg His Trp Lys Gly
Trp Leu Arg Arg Ile Gly Arg Arg Ile Glu Arg Val Gly Gln His

(2) INFORMATION FOR SEQ ID NO: 13:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:
Ala Ile Ala Lys Phe Ala Lys Lys Ala Leu Lys Ser Met Leu Ala
Leu Met Gly Glu Ala Val Gln Thr

(2) INFORMATION FOR SEQ ID NO: 14:
(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:
Ala Ile Ala Ile Phe Lys Arg Ile Ala Lys Ile Asn Phe Lys Ala
Leu Met Gly Glu Ala Val Gln Thr

(2) INFORMATION FOR SEQ ID NO: 15:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:
Ala Ile Ala Asn Phe Glu Arg Leu Met Lys Lys Leu Ile Trp Ala
Leu Met Gly Glu Ala Val Gln Thr

WHAT IS CLAIMED IS:

1. A protein having the sequence of any of SEQUENCE I.D. No. 1, SEQUENCE I.D. No. 2, SEQUENCE I.D. No. 3, SEQUENCE I.D. No. 4, SEQUENCE I.D. No. 5, SEQUENCE I.D. No. 6, SEQUENCE I.D. No. 7, SEQUENCE I.D. No. 8, SEQUENCE I.D. No. 9, SEQUENCE I.D. No. 10, SEQUENCE I.D. No. 11, SEQUENCE I.D. No. 12, SEQUENCE I.D. No. 13, SEQUENCE I.D. No. 14, or SEQUENCE I.D. No. 15.

2. A nucleotide sequence which codes for a protein according to Claim 1.

3. An RNA sequence according to Claim 2.

4. A DNA sequence according to Claim 2.

5. An expression cassette containing the DNA sequence of claim 4 operably linked to plant regulatory sequences which cause the expression of the DNA sequence in plant cells.

6. A bacterial transformation vector comprising an expression cassette according to Claim 5, operably linked to bacterial expression regulatory sequences which cause replication of the expression cassette in bacterial cells.

7. Bacterial cells containing as a foreign plasmid at least one copy of a bacterial transformation vector according to Claim 6.

8. Transformed plant cells containing at least one copy of the expression cassette of Claim 5.

9. Transformed cells according to Claim 8, further characterized in being cells of a monocotyledonous species.

10. Transformed cells according to Claim 9, further characterized in being maize, sorghum, wheat or rice cells.

11. Transformed cells according to Claim 8, further characterized in being cells of a dicotyledonous species.

12. Transformed cells according to Claim 11, further characterized in being soybean, alfalfa, rapeseed, sunflower, tobacco or tomato cells.

13. A maize cell or tissue culture comprising cells according to claim 10.

14. A transformed plant comprising transformed cells according to Claim 8.

15. A method for killing and inhibiting pathogenic microorganisms which are susceptible to a protein according to Claim 1, comprising introducing an antimicrobial amount of the protein into the environment of the pathogenic microorganisms.

16. A method for killing and inhibiting pathogens selected from F. graminearum, Fusarium moniliforme, F. oxysporum, A. flavus, P. medicaginis, Alternaria longipes, Colletototrichum graminicola, Verticillium albo-atrum, Phytophthora megasperme f.sp. glycinea, Macrophomina phaseolina, Diasporthe phaseolorum caulivor, Sclerotinia sclerotiorum, and Sclerotinia trifoliorum comprising introducing into the environment of the pathogenic microorganisms an antimicrobial amount of a protein according to Claim 1.

17. A method according to Claim 15 wherein the environment of the pathogen is the tissues of a living plant.

20. A method according to Claim 15 wherein the environment of the pathogen is the tissues of a living human or lower animal.